IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant	:	Hung et al.
Appl. No.	:	10/506,414
Filed	:	August 31, 2004
For	:	HUMAN SCHWANNOMA CELL LINE
Examiner	:	Hill, Kevin Kai
Group Art Unit	:	1633

DECLARATION UNDER 37 C.F.R §1.132

Mail Stop Amendment
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Dear Sir:

1. This Declaration is being submitted to demonstrate that prior to the present invention of immortalized human schwannoma cell line, method of its making and method of its using, persons skilled in the art were all confined to using human or mouse-originating primary Schwann cells, spontaneously immortalized mouse cell lines or immortalized rat cell lines. The claimed human cell line was and still is the first and the only available human immortalized schwannoma cell line.

2. I am an inventor on the above-identified patent application and am familiar with the specification and prosecution history.

3. I have extensive experience in the field of cell biology for many years. My Curriculum Vitae is attached as Exhibit A.

4. Although scientific research in the field of Schwann cells existed for many years, those skilled in the art had never had an opportunity to work on a human immortalized schwannoma cell line, as none existed until 2002. Researchers all over the world had had to rely on human primary cultures of these cells for NF2 research. Such cultures have been of limited value,

however, for the following reasons: 1) human Schwann cells are difficult to obtain, 2) very small numbers of cells can be obtained and cultured, 3) the cultures can be maintained for short periods of time and die quickly, and 4) fibroblasts often overgrow the culture. Other options available to the researchers were mouse-originating primary Schwann cells, spontaneously immortalized mouse cell lines or immortalized rat cell lines.

5. In 1990, Peden et al. published a paper (*Ann. N.Y. Acad. Sci.* 605:286-293), cited by the Examiner in the present application, which described methods of how to produce an immortalized rat Schwann cell line. Yet despite the long-felt need and the teaching of Peden et al. as far back as 1990, up until the present invention in 2002, the scientific community working in the field of Schwann cell and schwannoma cell research was either unmotivated to try to make immortalized human Schwann or schwannoma cells or unsuccessful in doing so. Consequently, scientists in this field still relied on working with primary cultures of human and mouse Schwann cells, immortalized rat Schwann cell line and mouse Schwannoma cell lines (see, for example, Thi et al. 1998, *J. Exp. Biol.* 201:851-860; Haynes et al. 1999, *Neurosci. Lett.* 271:155-158; Mambetisaeva et al. 1999, *J. Neurosci. Res.* 57:166-175; Detrait et al. 1999, *Neurosci. Lett.* 267:49-52), and spontaneously immortal mouse Schwann cell line (Nagano et al. 2001, *J. Neurochem.* 77:1486-1495; Shen et al. 2002, *J. Neurosci. Res.* 68:588-594, courtesy copies of these abstracts are attached for the Examiner's convenience).

6. Human Schwann cells were only available as primary cultures, and were very difficult to maintain (see Morrissey et al. 1995, *J. Neurobiol.* 28:171-189 and 190-201; Casella et al. 1996, *Glia* 17:327-338, abstracts attached)

7. Since the publication in 2002 of our manuscript describing the method of obtaining the first ever immortalized human schwannoma cell line, which was the basis of the present application, the Assignee of this application, House Ear Institute, obtained material transfer agreements from 18 research groups to deliver the sample of the claimed cell line (HEI-193) for their research. The list of these research groups included: Coriell Institute for Medical Research; Forham University; NexGenix Pharmaceuticals, LLC; University of Central Florida; Tufts-New England Medical Center; U.S. Davis; U. Texas; Marine BioLab; Cedars-Sinai Medical Center; Massachusetts General Hospital; UCSD; University of Cincinnati; U. of Hamburg; University of

Toledo (OH); Institute of Molecular and Cell Biology; Raven Biotechnologies, Inc.; and University of Erlangen-Nuremberg FAU Medical School. One of these research groups already published their results using the HEI193 cell line (Prabhakar et al. 2007, *Cancer Gene Therapy* 14:460-467).

8. The sheer number of such agreements attests to the long-felt need in the scientific community for an immortalized human schwannoma cell line as well as for the commercial success of the claimed cell line.

9. I declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful, false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or patent issuing therefrom.

Dated: 10-4-07 By: _____
Dr. Gene HUNG

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100107

BIOGRAPHICAL SKETCH

Provide the following information for the key personnel and other significant contributors in the order listed on Form Page 2.
Follow this format for each person. **DO NOT EXCEED FOUR PAGES.**

NAME	POSITION TITLE
Gene Hung	Director, Molecular and Experimental Pathology
eRA COMMONS USER NAME	

EDUCATION/TRAINING *(Begin with baccalaureate or other initial professional education, such as nursing, and include postdoctoral training.)*

INSTITUTION AND LOCATION	DEGREE *(if applicable)*	YEAR(s)	FIELD OF STUDY
Tian-jing Medical School	M.D.	1985	Medicine

A. Positions and Honors.

Positions and Employment

1985-1989 Residency, Sugical Pathology, TongRen Hospital, Beijing Capital Medical School
1989-1993 Fellow, Dept. of Pathology, USC School of Medicine
1993-1995 Postdoctoral Fellow, Gene Therapy Program, USC
1995-1996 Project Manager, Gene Therapy Program, USC
1996-2002 Clinical Assistant Professor, USC
1997-2002 Chief and Principal Scientist, House Ear Institute, Los Angeles, CA,
2002-2004 Chief and Director of Molecular Pathology, Arena Pharmaceuticals Inc. San Diego.CA
2004- Director of Molecular and Experimental Pathology, Isis Pharmaceuticals, Inc. San Diego. CA

B. Selected peer-reviewed publications (in chronological order).

1) Swayze EE, Siwkowski AM, Wancewicz EV, Migawa MT, Wyrzykiewicz TK, Hung G, Monia BP, Bennett CF. *Antisense oligonucleotides containing locked nucleic acid improve potency but cause significant hepatotoxicity in animals.* Nucleic Acids Res. 2007;35(2):687-700. Epub 2006 Dec 19.

2) Smith RA, Miller TM, Yamanaka K, Monia BP, Condon TP, **Hung G**, Lobsiger CS, Ward CM, McAlonis-Downes M, Wei H, Wancewicz EV, Bennett CF, Cleveland DW. *Antisense oligonucleotide therapy for neurodegenerative disease.* J Clin Invest. 2006 Aug;116(8):2290-6. Epub 2006 Jul 27.

3) Chadee DN, Xu D, **Hung G**, Andalibi A, Lim DJ, Luo Z, Gutmann DH, Kyriakis JM. *Mixed-lineage kinase 3 regulates B-Raf through maintenance of the B-Raf/Raf-1 complex and inhibition by the NF2 tumor suppressor protein.* Proc Natl Acad Sci U S A. 2006 Mar 21;103(12):4463-8. Epub 2006 Mar 13.

4) Fraenzer, J, Pan, H; Minimo L; Smith, G; Knauer D; **Hung, G** *Overexpression of the NF2 gene inhibits schwannoma cell proliferation through promoting PDGFR degradation* 2003. Int J Oncol

5) Kwon YJ, **Hung G**, Anderson WF, Peng CA, Yu H. Determination of infectious retrovirus concentration from colony-forming assay with quantitative analysis. J Virol. 2003 May; 77(10):5712-20.

6) Hung, G., Colton, J., Fisher, L., Ophenheimer, M., Faudoa, R., Slattery, W., Linthicum, F. *Immunohistochemistry study of Human Vestibular Nerve Schwannomas Differentiation* Glia 38:363-370, 2002

7) Linthicum FH Jr, Saleh ES, Hitselberger WE, Brackmann DE, **Hung G**. *Growth of postoperative remnants of unilateral vestibular nerve schwannoma: role of the vestibular ganglion.* J Otorhinolaryngol Relat Spec. 2002 Mar-Apr;64(2):138-42.

8) Lee, F, Linthicum, F, **Hung, G** *Proliferation potential in recurrent acoustic schwannoma following Gamma Knife radiosurgery versus microsurgery* The Laryngoscope 2002;112:948-950

9) **Hung G**, Li X, Faudoa R, Xeu Z, Kluwe L, Rhim JS, Slattery W, Lim D. *Establishment and characterization of a schwannoma cell line from a patient with neurofibromatosis 2.* Int J Oncol 2002 Mar;20(3):475-82

10) Bruder CE, Hirvela C, Tapia-Paez I, Fransson I, Segraves R, Hamilton G, Zhang XX, Evans DG, Wallace AJ, Baser ME, Zucman-Rossi J, Hergersberg M, Boltshauser E, Papi L, Rouleau GA, Poptodorov G, Jordanova A, Rask-Andersen H, Kluwe L, Mautner V, Sainio M, **Hung G**, Mathiesen T, Moller C, Pulst SM, Harder H, Heiberg A, Honda M, Niimura M, Sahlen S, Blennow E, Albertson DG, Pinkel D, Dumanski JP. *High resolution deletion analysis of constitutional DNA from neurofibromatosis type 2 (NF2) patients using microarray-CGH.*Hum Mol Genet. 2001 Feb 1;10(3):271-82.

11) Faudoa, R., Xue, Z., Lee, F., Baser, M, **Hung, G**. *Detection of NF2 mutations by an RNA mismatch cleavage method.* Human Mutation. 15: 474-478, 2000

12) **Hung, G.**, Baser, M., Faudoa, R., Xue, Z., Kluwe, L, Slattery, W., Brackmann, D., Lim, D. *Neurofibromatosis 2 (NF2) phenotypes and germ-line NF2 mutations determined by an RNA mismatch method and loss of heterozygosity analysis in NF2 schwannomas.* Cancer Genetics and Cytogenetics. 118: 167-168, 2000.

13) Chen, X., Zhang, D., Dennert, G., **Hung, G.**, Lee, A. E *Eradication of murine mammrry adenocarcinoma through HSVtk expression directed by the glucose-starvation inducible grp78 promoter* Breast Cancer and Treatment 59: 81-90, 2000

14) **Hung, G.**, Gazit, G., Chen, X., Anderson, W.F., Lee, A.S. *Use of the glucose starvation-inducible glucose- regulated protein 78 promoter in suicide gene therapy of murine fibrosarcoma.* Cancer Res, 1999, July 1; 59 (13):3100-6.

15) **Hung, G.,** Faudoa, R., Xue, Z., Yu, J., Lee, F., Baser, M., Brackmann, D., Hitselberger, W., Anderson, W.F., Lim, D. *Development of a novel in vitro system for the study of Neurofibromatosis Type Two.* Int. J. Oncol laryngealscope., March 14(3): p. 409-15.

16) **Hung, G.**, Skotzko, M.J., Chang, M., Parekh, D., Stain, S.C., Hall, F.L., Gordon, E.M., Anderson, W.F. (1997). *Intratumoral injection of a concentrated antisense cyclin G1 retroviral vector inhibits growth of undifferentiated carcinoma in nude mice.* Intl J of Ped Hema/Onc. Vol. 4: p.317-325.

17) Chen, D.S., Zhu, N.L., **Hung, G.**, Skozko, J.J., Hinton, Skozko, Tolo, V., Hall, F.L., Anderson, W.F., Gordon, E.M. (1997). *Retroviral vector-mediated transfer of an antisense cyclin G1 construct inhibits osteosarcoma tumor growth in nude mice.* Hum Gene Ther. 8 (14): p. 1667-74.

18) Press, M.F., Bernstein, L, Thomas, P.A., Meisner, L,F., Zhou, J.Y., Ma, Y, **Hung, G.**, Robinson, R.A., Harris, C., El-naggar, A., Slamon, D.J., Phillips, R.N., Ross, J.S., Wolman, S.R., Flom, K.J.(1997) *HER-2/neu gene amplification characterized by fluorescence in situ hybridization: poor prognosis in node-negative breast carcinomas.* J Clin Oncol., 15(8): p. 2894-904.

19) Press, M.F., Pike, M.C., **Hung, G.**, Zhou, J.Y., Ma, Y., George,J., Dietz-Band, J., James, W., Slamon, D.J., Batsakis, J.G. and et al. (1994). *Amplification and over expression of HER-2/neu in carcinomas of the salivary gland: correlation with poor prognosis.* Cancer Res., 54(21): p. 5675-82.

20) Press MF, **Hung G**, Godolphin W, Slamon DJ. (1994) *Sensitivity of HER-2/neu antibodies in archival tissue samples: potential source of error in immunohistochemical studies of oncogene expression..* Cancer Res. May 15;54(10):2771-7.

21) Press MF, Pike MC, Chazin VR, **Hung G**, Udove JA, Markowicz M, Danyluk J, Godolphin W, Sliwkowski M, Akita R, et al. (1993) *Her-2/neu expression in node-negative breast cancer: direct tissue quantitation by computerized image analysis and association of overexpression with increased risk of recurrent disease.* Cancer Res. Oct 15;53(20):4960-70.

C. Research Support

Ongoing Research Support

Co-Investigator on an Isis internal project which aim to character molecular mechanism of oligonucleotides accumulation and metabolism in kidney

PI on an Isis internal study of develop antisense drug applications for CNS disorders

Completed Research Support

US Army grant DAMD17-99-1-9491
The goals of this study were

1) To develop a novel in vitro system
2) To characterize NF2 gene tumor suppressor function
3) To establish a prove of concept for therapeutic interventions.

1: J Exp Biol. 1998 Mar;201(Pt 6):851-60.

Proliferation and differentiation properties of permanent Schwann cell lines immortalized with a temperature-sensitive oncogene.

Thi AD, Evrard C, Rouget P.

Laboratorie Biologie Moléculaire et Différenciation, Unité de Génétique Oncologique, CNRS-URA 1967, Insitut Gustave Roussy, Villejuif, France.

Permanent Schwann cells lines have been established in culture after stable transfection of newborn rat Schwann cells with the pJC-SVLTtsA vector, expressing a thermosensitive oncogene driven by the early promoter-enhancer region of the gliotropic GS/B variant of the papovavirus JC. The proliferation and differentiation of two clonal cell lines have been studied. The cells of these lines display the morphology of primary Schwann cells and express Schwann cell differentiation markers such as the S-100 protein, laminin, the low-affinity receptor to nerve growth factor and the glial fibrillary acidic protein. One of the lines is able to differentiate further. Indeed, in the presence of dorsal root ganglion neurones, the cells synthesize the myelin Po protein and are capable of some myelination, although to a lesser extent than secondary Schwann cells.

PMID: 9464965 [PubMed - indexed for MEDLINE]

1: Neurosci Lett. 1999 Aug 27;271(3):155-8.

Expression of neurofilament L-promoter green-fluorescent protein constructs in immortalized Schwann cell-neuron coculture.

Haynes LW, **Schmitz S**, **Clegg JC**, **Fooks AR**.

School of Biological Sciences, The University of Bristol, UK.
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The neurofilament L/68 protein (NF-L/68) gene is expressed in the immature Schwann cell phenotype but suppressed after myelin-formation. We have investigated conditions which regulate the activity of the NF-L/68 promoter in green fluorescent protein reporter constructs expressed in the immortal rat Schwann cell strain SCL4.1/F7 in coculture with neurons. Constructs expressed in a plasmid vector containing both the full-length promoter and the 3' proximal 107 bp sequence which includes the cyclic AMP response element (CRE), were active in SCL4.1/F7 cells, but were suppressed as the cells underwent spontaneous growth-arrest. Interaction of SCL4.1/F7 with axons accelerated downregulation of expression from both constructs, however expression of the full-length promoter continued in some cells until the onset of myelin-formation. Expression of the NFL/68 construct recommenced when demyelination was induced in culture by exposure to human sera from patients with paraproteinemic gammopathy. We have demonstrated a method to study the regulation of gene expression patterns in single Schwann cells interacting with neurons and shown that different promoter regions may be controlled by axon-related and -unrelated factors.

PMID: 10507692 [PubMed - indexed for MEDLINE]

1: J Neurosci Res. 1999 Jul 15;57(2):166-75.

Multiple connexin expression in peripheral nerve, Schwann cells, and Schwannoma cells.

Mambetisaeva ET, **Gire V**, **Evans WH**.

Department of Medical Biochemistry, University of Wales College of Medicine, Heath Park, Cardiff, Wales, United Kingdom.

Myelinating Schwann cells express the gap junction protein, connexin (Cx)32, which is present at the nodes of Ranvier and Schmidt-Lantermann incisures (Bergoffen et al. [1993] Science (Wash.) 262:2039-2042). Following peripheral nerve injury, other members of the connexin gene family are also expressed (Chandross et al. [1996a] Mol. Cell. Neurosci. 7:501-518). This study surveys the connexin(s) expressed by rat sciatic nerve, cultured Schwann cells, and a mouse Schwannoma (TR6 Bc1) cell line. Reverse transcriptase-polymerase chain reaction (RT-PCR) amplification revealed a constitutive expression of mRNA encoding Cx32 and 43 but not Cx26, 37, 40, 45, and 46 in sciatic nerve. Mitogenic stimulation of cultured Schwann cells expressing Cx32 also resulted in the appearance of Cx43 mRNA. Schwannoma cells expressed exclusively Cx43 mRNA. These results were confirmed by Northern blot analysis. Functional gap junctions in cultured Schwann and Schwannoma cells were shown by analysis of the intercellular transfer of Lucifer yellow, although the coupling between primary Schwann cells was weak or undetectable. Treatment of primary Schwann cells with mitogens resulted in extensive dye coupling. An immunohistochemical study of adult sciatic nerve sections demonstrated Cx32 immunoreactivity at the nodes of Ranvier and in Schwann cell bodies. Lower intensity staining of Cx43 along the myelin sheath and Schwann cell bodies was also observed. Indirect immunofluorescent studies of Schwann cells treated with mitogens showed characteristic punctate cell surface staining of Cx43; Cx32 staining was detected mainly intracellularly. These results lead to the conclusion that in addition to the expression of Cx32 by normal adult sciatic nerve, low amounts of Cx43 protein are also present. The implications of the expression of two connexins by Schwann cells in Charcot-Marie-Tooth X-linked disease, a demyelinating peripheral neuropathy, are discussed.

1: Neurosci Lett. 1999 May 21;267(1):49-52.

Expression of integrins by murine MSC80 Schwann cell line: relationship to cell adhesion and migration.

Detrait E, Laduron S, Meremans V, Baron-Van Evercooren A, van den Bosch de Aguilar P, Knoops B.

Department of Biology, Université Catholique de Louvain, Louvain-la-Neuve, Belgium.

Schwann cells (Sc) are one of the most important factors promoting regeneration of both the peripheral and the central nervous system. They provide a permissive environment for neurite outgrowth and the making of this environment requires interactions between Sc and extracellular matrix proteins that are mediated via integrin receptors. This study characterized, by immunoprecipitation, the integrins expressed by the mouse MSC80 Sc line. Our results showed that MSC80 Sc expressed alpha1beta1, alpha5beta1 and alpha6beta1 integrins as well as the alpha v-subunit associated with an unidentified 80-90 kDa beta-subunit. Adhesion and migration assays revealed a hierarchy of protein influences that are dependent upon the type of cellular behaviour. Integrin expression correlated with MSC80 Sc line adhesion and migration on extracellular matrix proteins. The MSC80 Sc line expressed a pattern of integrins which allowed adherence on vitronectin and collagen IV, and faster migration on merosin and laminin. As the integrin pattern and the behaviour of MSC80 on ECM were similar to primary Sc, MSC80 are a potential abundant source of Sc for further in vitro and in vivo experiments.

PMID: 10400246 [PubMed - indexed for MEDLINE]

1: J Neurochem. 2001 Jun;77(6):1486-95.

Cytokine-induced cell death in immortalized Schwann cells: roles of nitric oxide and cyclic AMP.

Nagano S, **Takeda M**, **Ma L**, **Soliven B**.

Department of Neurology and Communication on Neurobiology, The Brain Research Institute, The University of Chicago, Illinois 60637, USA.

Tumor necrosis factor-alpha and interferon-gamma are pleiotropic cytokines that regulate Schwann cell responses during injury and inflammatory demyelination. We have previously shown that cyclic AMP (cAMP)-elevating agents decrease the demyelination and Wallerian degeneration in experimental allergic neuritis. In this study, we examined the role of cAMP in cytokine-mediated signaling in a spontaneously immortal Schwann cell clone (iSC). We found that tumor necrosis factor-alpha and interferon-gamma exert synergistic inhibitory action on Schwann cell viability via the production of nitric oxide (NO) and ceramide (cer). Furthermore, we found that: (i) NO synthase inhibitors attenuate the cytokine-induced cer accumulation and cell death indicating that NO acts upstream of cer; and (ii) cytokine-induced cell death is decreased in iSCs pretreated continuously for 48-72 h with forskolin, an activator of adenylate cyclase. Although forskolin modulates the phosphorylation of ERKs and Akt, it decreases the susceptibility of iSC to cytokines via a separate mechanism operating after NO induction and before cer accumulation. We propose that the protective effect of cAMP-elevating agents in experimental allergic neuritis may be mediated in part via modulation of Schwann cell responses to cytokines.

PMID: 11413232 [PubMed - indexed for MEDLINE]

1: J Neurosci Res. 2002 Jun 1;68(5):588-94.

Establishment and characterization of spontaneously immortalized Schwann cells from murine model of globoid cell leukodystrophy (twitcher).

Shen JS, **Watabe K**, **Meng XL**, **Ida H**, **Ohashi T**, **Eto Y**.

Department of Gene Therapy, Institute of DNA Medicine, The Jikei University School of Medicine, Tokyo, Japan.

The twitcher mouse is a murine model of human globoid cell leukodystrophy (GLD; Krabbe disease) caused by a genetic defect in the activity of galactosylceramidase (GALC). An accumulation of cytotoxic metabolite, galactosylsphingosine (psychosine), in myelin forming cells (oligodendrocytes and Schwann cells) of the twitcher mouse as well as patients with GLD has been suggested to cause dysfunction of these cells and subsequent demyelination in the central and peripheral nervous system. To investigate further the cellular pathomechanism of GLD, we established spontaneously immortalized Schwann cell lines from the twitcher mouse. Long-term cultures of Schwann cells derived from dorsal root ganglia and consecutive peripheral nerves of 3-week-old twitcher mice were maintained for 6 months, and spontaneously developed colonies were expanded further and characterized. One of the cell lines, designated TwS1, showed distinct Schwann cell phenotypes, was passaged twice a week and maintained for over 10 months without phenotypic alterations. The TwS1 cells had a nonsense mutation in the GALC genome, and showed markedly reduced GALC activity and elevated psychosine levels. Ultrastructurally, varieties of cytoplasmic inclusions were demonstrated in TwS1 cells. When TwS1 cells were infected with a retrovirus vector encoding GALC, GALC activity was markedly increased and psychosine levels were significantly decreased. These immortalized Schwann cells can be useful in studies on the nervous system lesions in GLD. Copyright 2002 Wiley-Liss, Inc.

PMID: 12111848 [PubMed - indexed for MEDLINE]

1: J Neurobiol. 1995 Oct;28(2):171-89.

Links

Human Schwann cells in vitro. I. Failure to differentiate and support neuronal health under co-culture conditions that promote full function of rodent cells.

Morrissey TK, **Bunge RP**, **Kleitman N**.

Miami Project to Cure Paralysis, University of Miami School of Medicine, Florida 33136, USA.

Schwann cells (SCs) play critical roles in regeneration after injury to the peripheral nervous system and can also induce axonal regeneration in the central nervous system. Transplantation of purified SCs into sites of neural injury in rodents has confirmed the remarkable ability of these cells to promote axonal regrowth, suggesting that human application of SC transplantation could be valuable. In this report, we have compared the functional capacities of SCs derived from adult human and rodent nerves by of SCs derived from adult human and rodent nerves by maintaining SCs from these two sources in culture with sensory neurons. We noted that techniques commonly in use for maintaining pure rat SC populations are not sufficient to sustain populations of human SCs free of fibroblasts. In these co-cultures, human SCs express a limited profile of characteristic behaviors and they proliferate more slowly than rat SCs in response to axonal contact. Slow SC proliferation, relative to that of contaminating fibroblasts, leads to a high proportion of fibroblasts in the cultures. After 3 to 4 weeks of co-culture with neurons, human SCs express extracellular matrix molecules, but only partially ensheathe axons, whereas rat SCs differentiate, form basal lamina, and ensheathe or myelinate axons. Co-culture of sensory neurons with human (but not rat) SC preparations (or conditioned medium therefrom) leads to a progressive neuronal atrophy characterized by shrinking neuronal cell bodies and a decrease in the density of the neurite network in the culture dish. As the divergent effects of human and rat SCs on neuronal health were also observed in co-cultures with human sensory neurons, these effects reflect differences between the rat and human-derived SC populations, rather than a species mismatch between SCs and neurons. The marked differences in behavior observed between rat and human SCs derived by the same methods requires further exploration if human-derived SCs are to be considered in the treatment of disease.

Related Links

- Human Schwann cells in vitro. II. Myelination of sensory axons following extensive purification and heregulin-induced expansion. [J Neurobiol. 1995]
- Functional status influences the ability of Schwann cells to support adult rat retinal ganglion cell survival and axonal regrowth. [Exp Neurol. 1989]
- N-cadherin mediates axon-aligned process growth and cell-cell interaction in rat Schwann cells. [J Neurosci. 2002]
- Cultured Schwann cells assemble normal-appearing basal lamina only when they ensheathe axons [Dev Biol. 1989]
- The functional characteristics of Schwann cells cultured from human peripheral nerve after transplantation into a gap within the rat [J Neurosci. 1994]

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In a companion article we report experiments that define culture conditions more effective in promoting human SC function in vitro.

PMID: 8537823 [PubMed - indexed for MEDLINE]

1: J Neurobiol. 1995 Oct;28(2):190-201.

Links

Human Schwann cells in vitro. II. Myelination of sensory axons following extensive purification and heregulin-induced expansion.

Morrissey TK, **Kleitman N**, **Bunge RP**.

Miami Project to Cure Paralysis, University of Miami School of Medicine, Florida 33136, USA.

Co-culture conditions are well established in which Schwann cells (SCs) derived from immature or adult rats proliferate and form myelin in response to contact with sensory axons. In a companion article, we report that populations of adult-derived human Schwann cells (HASCs) fail to function under these co-culture conditions. Furthermore, we report progressive atrophy of neurons in co-cultures containing populations of either human fibroblasts or HASCs (which contain both SCs and fibroblasts). Two factors that might account for the insufficiency of the co-culture system to support HASC differentiation are the failure of many HASCs to proliferate and the influence of contaminating fibroblasts. To minimize fibroblast contamination of neuron-HASC co-cultures, we used fluorescence-activated cell sorting to highly purify HASC populations (to more than 99.8%). To stimulate expansion of the HASC population, a mitogenic mixture of heregulin (HRG beta 1 amino acid residues 177-244; 10 nM), cholera toxin (100 ng/mL), and forskolin (1 microM) was used. When these purified and expanded HASCs were co-cultured with embryo-derived rat sensory neurons, neuronal shrinkage did not occur and after 4 to 6 weeks some myelin segments were seen in living co-cultures. This myelin was positively identified as human by immunostaining with a monoclonal antibody specific to the human peripheral myelin protein P0 (antibody 592). Although this is the first reported observation of myelination by HASCs in tissue culture, it should be noted that myelination occurred more slowly and in much less abundance than in comparable cultures containing adult rat-derived SCs. We anticipate that further refinements of the HASC co-culture system that enhance myelin formation will provide insights into important aspects of human SC biology and provide new opportunities for studies of human peripheral neuropathies.

PMID: 8537824 [PubMed - indexed for MEDLINE]

1: Glia. 1996 Aug;17(4):327-38.

Links

Improved method for harvesting human Schwann cells from mature peripheral nerve and expansion in vitro.

Casella GT, **Bunge RP**, **Wood PM**.

Miami Project to Cure Paralysis, University of Miami School of Medicine, Florida 33136, USA.

The use of cellular prostheses containing large populations of Schwann cells (SC) has been proposed as a future therapeutic approach in the repair of neural tissue. We have sought to define an efficient protocol for the harvest and expansion of human SC from mature human peripheral nerve. We evaluated SC proliferation occurring within fresh explants and studied the relationship between certain parameters (cell yield, purity, and rate of SC proliferation) and the conditions of maintenance of nerve explants prior to dissociation. In addition, we studied SC proliferation after dissociation in a variety of conditions. We observed that SC within explants divide at a low rate during the first 3 weeks following explantation; this proliferation falls to near zero during the fourth week. The cell yield, SC purity, and proliferation rate following dissociation were all increased when nerve explants were exposed to heregulin/ forskolin for 2 weeks prior to dissociation. Electron microscopic analysis showed that heregulin/forskolin exerted trophic effects on SC within explants. Following dissociation, SC growth in heregulin/forskolin-containing medium was more rapid on laminin or collagen than on poly-L-lysine. These results provide new insights into human SC biology and suggest several procedural improvements for harvesting and expanding these cells. The new method we describe shortens our previous procedure by 4-6 weeks and provides a 30-50-fold increase in the number of SC obtained relative to the earlier procedure.

PMID: 8856329 [PubMed - indexed for MEDLINE]

Cancer Gene Therapy (2007) 14, 460–467

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ORIGINAL ARTICLE

Treatment of Implantable NF2 Schwannoma Tumor Models with Oncolytic Herpes Simplex Virus G47Δ

S Prabhakar[1,2], SM Messerli[1,2,5], AO Stemmer-Rachamimov[3], T-C Liu[4], S Rabkin[4], R Martuza[4] and XO Breakefield[1,2]

[1]*Molecular Neurogenetics Unit, Departments of Neurology and Radiology, Massachusetts General Hospital, Charlestown, MA, USA;* [2]*Neuroscience Program, Harvard Medical School, Boston, MA, USA;* [3]*Molecular Neuro-Oncology Laboratory and Department of Pathology, Massachusetts General Hospital, Boston, MA, USA and* [4]*Department of Neurosurgery, Massachusetts General Hospital, Boston, MA, USA*

Schwannomas are benign tumors composed of dedifferentiated Schwann cells that form along peripheral nerves causing nerve compression often associated with pain and loss of function. Current surgical therapy involves total or subtotal surgical removal of the tumor, which may cause permanent nerve damage. In the present study, we explore an alternate means of therapy in which schwannomas are injected with a replication-conditional herpes simplex virus (HSV) vector to shrink the tumor through cell lysis during virus propagation. The oncolytic vector used, G47Δ, has deletions in *HSV* genes, which allow it to replicate selectively in dividing cells, sparing neurons. Two schwannoma cell lines were used to generate subcutaneous tumors in nude mice: HEI193, an immortalized human line previously established from an NF2 patient and NF2S-1, a newly generated spontaneous mouse line. Subcutaneous HEI193 tumors grew about ten times as fast as NF2S-1 tumors, and both regressed substantially following injection of G47Δ. Complete regression of HEI193 tumors was achieved in most animals, whereas all NF2S-1 tumors resumed growth within 2 weeks after vector injection. These studies provide a new schwannoma model for testing therapeutic strategies and demonstrate that oncolytic HSV vectors can be successfully used to shrink growing schwannomas.
Cancer Gene Therapy (2007) **14,** 460–467. doi:10.1038/sj.cgt.7701037; published online 16 February 2007

Keywords: neurofibromatosis type 2; oncolytic virus; HSV; mouse schwannoma; schwannoma cell line; human schwannoma

Introduction

Schwannomas arise through unregulated overgrowth of Schwann cells, which normally serve to form a protective sheath around peripheral nerves.[1,2] Schwannomas can arise during development, as Schwann cells move out along axons and begin myelination, or later in life when Schwann cells dedifferentiate and commence proliferation in response to nerve injury.[3] In fact, Schwann cells in damaged nerves share many features with Schwann cells in development.[4] Although schwannomas are almost always benign, they cause clinical symptoms by continued growth and nerve compression. Schwannomas typically result from loss of the neurofibromatosis 2 tumor suppressor gene (*NF2*), but can also arise through other genetic mechanisms. In *NF2*, loss of one *NF2* allele is hereditary, with the loss of the second, wild-type allele occurring as a somatic mutation. Other hereditary syndromes in which patients develop multiple schwannomas include schwannomatosis, of unidentified genetic etiology,[5] and Carney's complex caused by loss of the tumor suppressor gene encoding the R1 alpha regulatory subunit of protein kinase A.[6] Schwannomas may also occur in the absence of a hereditary syndrome owing to a localized somatic cell mutation of the *NF2* gene. The *NF2* gene-encoded protein, merlin or schwannomin,[7,8] links the actin cytoskeleton with membrane proteins involved in signal transduction.[3]

Schwannomas of the eighth vestibular nerve are often associated with hearing loss, whereas schwannomas of other cranial nerves or peripheral nerves may cause chronic pain, loss of sensation and paralysis. When schwannomas become very large, compression of adjacent structures, brain stem, spinal cord peripheral nerves and organs, may occur.[9] Particularly challenging to treat by surgery are cranial nerve schwannomas owing to potential brain stem or cranial damage. Although schwannomas can be life-threatening because of their size and location, these tumors are usually not malignant and, therefore, reductions in volume alone can be highly beneficial, as complete surgical resection is not always possible or

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may be associated with serious complications. Treatment strategies need to focus on reducing the size of schwannomas while sparing associated nerves.

One obstacle to the development of new therapeutic interventions for schwannomas is the lack of appropriate experimental animal models for convenient testing. Although several mouse models of NF2 schwannomas have been generated, these are limited by a long time interval to tumor formation, random location of tumors within the animals and the difficulty in assessing changes in tumor size over time in living animals. For example, two genetic mouse models have been developed by Dr Giovannini. Transgenic mice expressing a dominant mutant form of merlin (found in human patients) under control of the Schwann cell-specific P0 promoter develop multiple schwannomas and Schwann-cell hyperplasias.[10] However, these lesions can take over a year to form, and are in sporadic locations making it difficult to monitor growth by *in vivo* imaging and to access tumors for intervention.[11] An NF2 floxed mouse model has also been generated and characterized.[12] When homozygous $Nf2^{flox2/flox2}$ mice are crossed with transgenic mice bearing Cre under the P0 promoter, offspring develop both benign and malignant Schwann cell tumors from 10 months of age or later predominantly within the spinal ganglia, with lower frequency in peripheral nerves. Meningiomas can also be elicited in these Nf2 floxed mice by injection of adenovirus vectors encoding Cre into the neonatal mouse brain.[13] When these P0Cre × Nf2 floxed mice are also hemizygous for the tumor suppressor *p53*, they develop multiple malignant peripheral nerve sheath tumors within the first 2 months of age.[14] These tumors are accessible, but again they occur in random locations and are not typical of most schwannomas found in human patients, and the mice die within a few months of age. Xenograft models have also been generated by implanting fragments of schwannoma tissue (surgical material from patients) subcutaneously into nude mice.[15] However, this method had very low yield with schwannoma tissues from only two patients out of 12 successfully giving rise to schwannoma xenografts, which showed consistent growth over a period of months (with a rate of growth similar to spontaneous lesions in *NF2* transgenic mice). These tumors had the histological features of peripheral nerve sheath tumors and showed diffuse S100 immunopositivity. Finally, although tumor fragments could be placed in sites accessible to measurement and injection, they grew very slowly and in some cases elicited an inflammatory response over months even in nude mice. This led us to explore schwannoma cell lines, which could be grown in culture and implanted into nude mice with a high rate of tumor formation.

Therapeutic treatment of schwannomas typically involves surgical resection, which may result in permanent nerve damage. In a previous study, we found that schwannoma cells from Nf2 transgenic mice and human schwannoma xenograft implants are highly infectable with HSV vectors.[15] We used a recombinant, oncolytic vector, G47Δ, with mutations in viral genes that allow it to replicate selectively in tumor cells.[16,17] G47Δ is deleted for gamma 34.5 genes and the *ICP6* gene encoding the large subunit of ribonucleotide reductase (with insertion of the *lacZ* gene), both of which promote selective virus replication in tumor cells[18] as well as being deleted for the *ICP47* gene, which is involved in blocking presentation of viral antigens through the major histocompatibility complex class I (MHC I) pathway.[19] This oncolytic virus was effective at reducing tumor size in both the Nf2 transgenic and human schwannoma xenograft models.[15]

In the present study, we evaluate the ability of the oncolytic G47Δ virus to reduce effectively the size of subcutaneous tumors derived from two schwannoma cell lines; a human schwannoma line, HEI193, derived by Hung *et al.*[20] through immortalization of NF2 tumor cells in culture by infection with a retrovirus vector encoding human papilloma virus (HPV) *E6-E7* genes, and a mouse schwannoma line, NF2S-1, generated in the present study by culturing cells from a tumor formed in nude mice at the site of implantation of a human schwannoma fragment from an NF2 patient. Both schwannoma cell lines expressed S100 and were highly infectable with G47Δ virus. Subcutaneous tumors formed by implantation of these cells in nude mice regressed substantially following injection of G47Δ, with complete regression seen for most HEI193 tumors.

Materials and methods

Cell culture

Human HEI193 schwannoma cells were obtained from the House Ear Institute.[20] Mouse NF2S-1 schwannoma cells were generated from a spontaneous tumor that formed in *nu/nu* mice (NCI), following subcutaneous implantation of mediastinal schwannoma tissue from an NF2 patient (as per the clinical diagnosis of the primary physician), which was resected at the time of clinically indicated surgery under IRB approval. Three months after implantation, a small tumor of about 3 mm^3 at the site of implantation was removed. Tumor dissociation was carried out overnight at 37 °C in 20 ml of L15 medium (Gibco, Grand Island, NY) with 1.25 U of dispase (Gibco)/ml, 0.05% collagenase (Sigma, St Louis, MO) and 50 μg/ml gentamicin (Gibco). Cells were collected by centrifugation and resuspended in Dulbecco's modified Eagle's medium (DMEM, Cellgro, Herndon, VA) containing serum-free N-2 Supplement (Gibco) with 2 μM forskolin (Calbiochem, San Diego, CA), 14 ng/ml of recombinant human glial growth factor (rhGGF2, Sigma), 50 μg/ml gentamicin, 10% fetal bovine serum (FBS, Sigma) and 100 μg/ml penicillin/streptomycin (P/S, Cellgro), and plated onto poly-L-lysine-coated dishes (35 mm). All cells were grown at 37 °C in a 95% air/5% CO_2 atmosphere.

For karyotyping, schwannoma cells were harvested and metaphase spreads were stained by Giemsa-Trypsin-Giemsa banding and evaluated by the Dana Farber/Harvard Cancer Center Cytogenetics Core Laboratory at Brigham and Women's Hospital.

Generation and propagation of G47Δ

G47Δ was propagated and titered as described in Todo *et al.*[19] Briefly, Vero cells were grown in DMEM with 10% FBS, P/S and 500 μg/ml G418 (Geneticin, Gibco) and infected with G47Δ at a multiplicity of infection (MOI) = 0.02–0.03 plaque forming units (pfu)/cell for 90 min, then the viral inoculum was removed and DMEM containing 2% heat-inactivated FBS (Sigma) and 1:2000 human immunoglobin G (IgG) (BayGam/Bayer, Elkar, IN) was added. Two or 3 days later, when infected cells were rounded and refractile, they were harvested by centrifugation. Cell pellets were resuspended in a 1:1 mix of media and 150 mM NaCl/20 mM Tris (pH 7.5), and the cell suspension was lysed by three cycles of freeze-thawing. Following centrifugation (20 000 R.P.M., Beckman Centrifuge Rotor SW 28), progeny virus was titered on Vero cells, as described,[19] with typical titers of 2.5×10^8 pfu/ml.

To determine the burst size of viral progeny in schwannoma cells, HEI-193 and NF2S-1 cells were infected with G47Δ at approximately 1 pfu/cell and virus harvested and titered 1 day later. Cells were seeded into 12-well plates (1×10^5 cells/well), infected at 70–80% confluency, and the inoculum removed after 2 h and replaced with media (DMEM/1% FBS). Cells and medium were harvested at the indicated times postinfection, processed with three freeze–thaw cycles, sonicated, and titered on Vero cells. Experiments were repeated at least three times for each line in duplicate.

Generation and treatment of tumors

For the establishment of schwannomas, 5×10^6 cells, either HEI193 or NF2S-1 was suspended in 100 μl-reduced serum media (Opti-MEM 1, Gibco), mixed with 100 μl of Matrigel (1:1 ratio; BD Matrigel™ Matrix HC, BD Biosciences, Bedford, MA) and implanted subcutaneously into one flank of immunodeficient mice (*nu/nu*, NCI). When tumor growth was apparent (approximately 1 week after the injection), tumor volumes were measured once/twice a week with external calipers, and the volume was calculated as $(4\pi/3)(\text{width}/2)2(\text{length}/2)$.[21] When the tumors reached a size of 100 mm^3 (baseline day 1) the animals were randomly divided into two groups of six animals each. One group was injected with 2×10^7 pfu G47Δ diluted in 0.9% sodium chloride (1:3) (Abbott Laboratories, North Chicago, IL) in a total volume of 120 μl over 5 min on baseline day 7 and 13. A control group was injected with 120 μl PBS/saline (1:1) into each tumor at the same time points.

Histology and immunocytochemistry

Following treatment with G47Δ or vehicle, animals were killed under heavy anesthesia with ketamine (200 mg/kg)/xylazine (50 mg/kg) mix followed by transcardial perfusion with ice cold 0.1 M PBS (pH 7.4), then 4% buffered paraformaldehyde in PBS. Tumors were harvested, postfixed overnight, transferred to 30% sucrose solution and stored at 4 °C until they sank. Samples were sectioned from a cryostat at 30 μm and mounted on charged, precleaned Probe-On Plus Microscope slides (Fisher Scientific, Pittsburgh, PA). Hematoxylin and eosin (H&E) staining was performed on tissue sections, as described previously.[11]

For immunostaining of tissue sections, tissue was deparaffinized and hydrated, as described previously.[11] Sections were then permeabilized in 0.1% sodium azide/

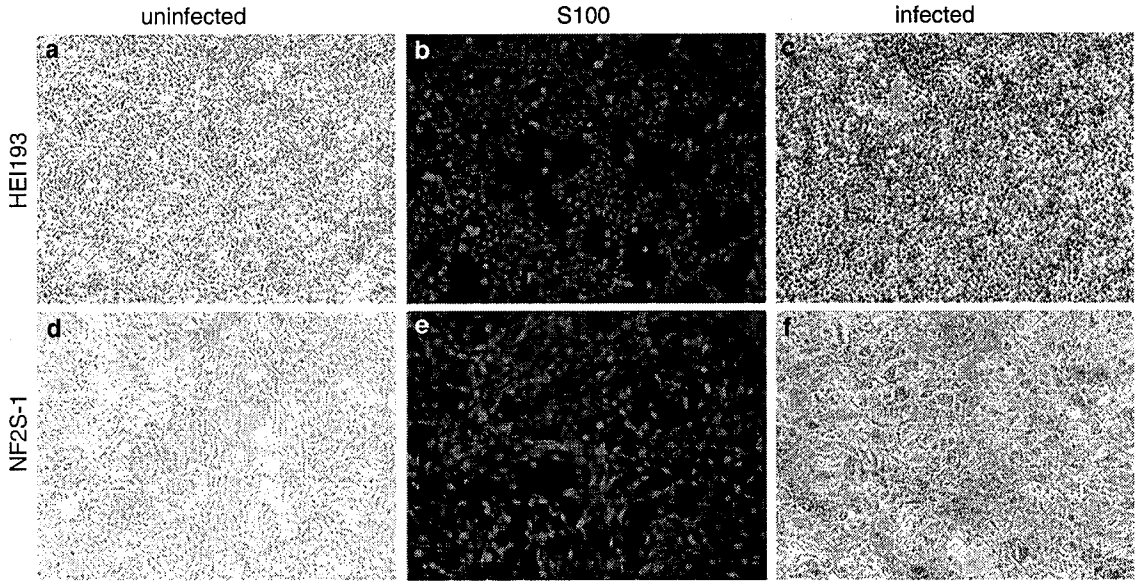

Figure 1 Phenotype and infectability of HEI193 and NF2S-1 cells. (**a** and **d**) Both cell types formed a confluent monolayer, with HEI193 cells being more uniform in size than NF2S-1 cells. (**b** and **e**) All cells in both lines stained robustly for the Schwann cell marker, S100 by immunocytochemistry. (**c** and **f**) At an MOI of 1.0, most cells of both lines were infected with G47Δ virus as revealed by staining for *lacZ* encoded in the virus 24 h postinfection. Magnification = × 10.

30% hydrogen peroxide for 20 min, washed 3 × with PBS and treated with blocking buffer (10% goat serum in 1% BSA and 0.1% Triton X-100 diluted in 1 × PBS) for 2 h. Staining for S100 using a polyclonal rabbit anti-cow S100 antibody (Dako, Glostrun Denman, Carpinteria, CA) was performed, as described previously.[11] Parallel sections were stained with the rabbit anti-β-galactosidase polyclonal antibody (Chemicon International, Temecula, CA) diluted 1:200 in 1% goat serum and then applied to tissue sections overnight at 4 °C. Other parallel sections were stained with monoclonal mouse antibody to glial fibrillary acidic protein (GFAP, Dako, Carpintera, CA) diluted 1:1000 in 1% BSA and applied to tissue sections overnight at 4 °C. Following the primary antibody incubation, sections were washed 3 × with PBS. The secondary goat anti-rabbit IgG-antibody (Alexa Fluor 594, Molecular Probes, Eugene, OR) for β-galactosidase stained slides was used at a dilution of 1:1500 and the secondary biotinylated horse anti-mouse antibody at a dilution of 1:2000 for 30 min at room temperature. Following incubation with the secondary antibody, sections were rinsed 3 × in PBS and dehydrated through grades of alcohol before being mounted with glass coverslips and fluorescent mounting medium (Dako).

Results

Characterization of schwannoma cell lines

The human schwanomma cell line used in these studies, HEI193, was originally generated by infection with a retrovirus vector encoding the immortalizing genes *E6-E7* from HPV and characterized by Hung *et al.*[20] The mouse schwannoma cell line, NF2S-1, was generated by injecting fragments of a schwannoma tumor from an NF2 patient subcutaneously into a nude mouse. When a tumor formed 3 months later (about 3 mm^3 in diameter) and the cells from it were placed in culture, they showed a mouse karyotype and a schwannoma phenotype. Both cell lines showed strong immunocytochemical staining for the Schwann cell marker S100 and both were highly infectable with G47Δ virus, which encodes *lacZ* (Figure 1). Properties of these two cell lines in culture are compared in Table 1. Karyotypic analysis indicated a chromosomal number of 67 human chromosomes (in 10/10 metaphases examined) for HEI193 and 43 mouse chromosomes for NF2S-1 (in 9/10 metaphases with the other one having 44 chromosomes). The previously reported karyotype of HEI193 was a modal chromosome number of 37 with about 25% of cells being tetraploid (presumably about 92 chromosomes[20]), and thus, during passage in our

Table 1 Properties of Schwannoma cell lines

Properties	*HEI193*	*NF2S-1*
Chromosome number	67	43
Species	Human	Mouse
Generation time (h)	12	24
Cells infected (%) (MOI = 1.0)	>90	>90
Burst size (pfu/cell)	2.3	0.9

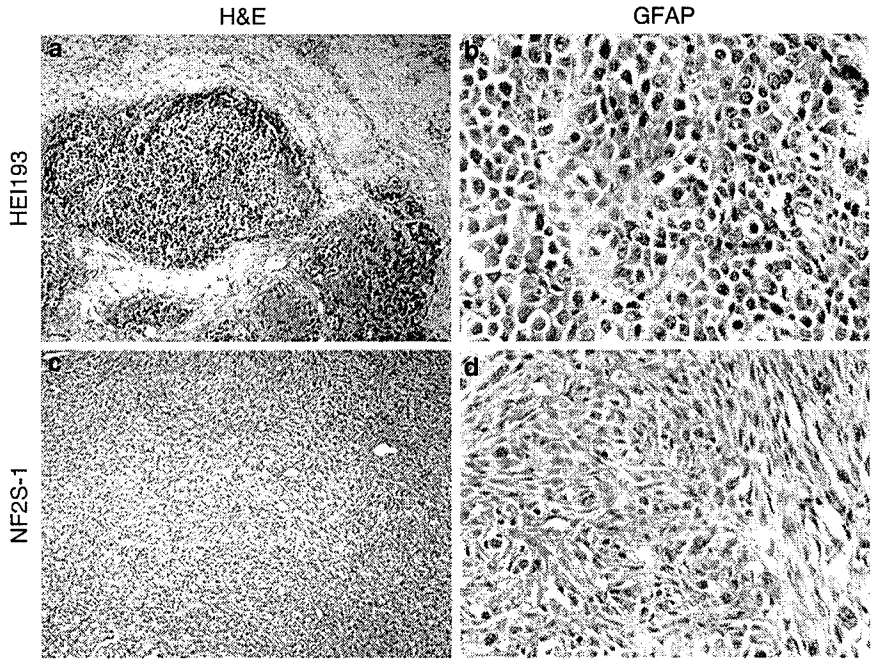

Figure 2 Pathological staining of schwannoma tumors. HEI193 and NF2S-1 tumors were removed after 25–35 days of growth after perfusing animals, fixed and mounted on paraffin sections. HEI193 (**a**) and NF2S-1 (**c**) tumors showed the characteristic cellular morphology of schwannoma tumors by H&E staining (magnification = × 10). Immunostaining for GFAP revealed immunonegativity for HEI193 tumors (**b**), whereas NF2S-1 tumors had many immunopositive cells (**d**). Magnification = × 40.

laboratory, the chromosomal number has increased. The HEI193 cells grew twice as rapidly as NF2S-1 cells with generation times of 12 and 24 h, respectively. Both lines proved highly infectable with the HSV G47Δ virus infecting >90% of HEI193 and NF2S-1 cells at an MOI of 1 pfu/cell. G47Δ was able to propagate in both cell types to a limited extent, HEI193 cells produced on average 2.3 pfu/cell and NF2S-1 cells 0.9 pfu/cell at 24 h after infection at an MOI of 1.

Growth and treatment of schwannomas xenografts

HEI193 and NF2S-1 cells were implanted subcutaneously into the flanks of nude mice with matrigel and tumor growth was evident after 1 week. Tumors were removed 25–35 days after implantation and histologically analyzed using H&E staining and GFAP immunostaining. The HEI193 human schwannoma was defined histologically as a poorly differentiated malignant tumor with a nodular growth pattern. Tumor cells were large and polymorphic and immunonegative for GFAP (Figure 2a and b). The NF2S-1 mouse schwannoma was composed of spindled cells, arranged in intersecting fascicles and was immunopositive for GFAP, consistent with Schwann cell origin (Figure 2c and d). The growth rate of these schwannomas in nude mice was monitored by the external calipers. After an initial delay, untreated HEI193 tumors grew rapidly at the rate of $131.95 \pm 11.4\,mm^3$/day ($n = 12$; Figures 3a and 5a). NF2S-1 tumors grew at about one-tenth that speed, at a rate of $17.37 \pm 1.37\,mm^3$/day ($n = 12$; Figures 3a and 5b). When tumors reached $100\,mm^3$ in size, they were injected directly with vehicle or G47Δ vector (2×10^7 pfu) at two successive times, after 5–7 days. Both tumors showed a progressive decrease in volume within 5–7 days after the first vector injection with a significant volume reduction starting at 2 weeks after the first virus injection (Figure 3a and b). The rate of reduction of HEI193 tumors after the first injection was about $70\,mm^3$/day ($n = 12$; Figures 3a and 5a) to 15% of the volume at time of injection after 2 weeks, that for NF2S-1 tumors was about $20\,mm^3$/day ($n = 12$; Figures 3b and 5b) to 30% of volume at time of injection after 3 weeks. Both types of treated tumors, removed 2 weeks after the last virus injection, showed positive immunocytochemistry for S100 (Figure 4a and c) and some continuing virus replication as indicated by a few *lacZ*-positive cells within the tumor (Figure 4b and d).

To determine whether G47Δ could eliminate tumors, subcutaneous HEI193 (Figure 5a) and NF2S-1 (Figure 5b) tumors were established and injected as above with vector when they reached a size of $100\,mm^3$ on successive days 7 and 10 (HEI193) or 14 and 21 (NF2S-1). Both types of G47Δ-treated tumors regressed in size. A paired *t*-test indicated HEI193 tumors were significantly smaller than uninjected tumors 10 days following initial virus injection ($P < 0.002$) and NF2S-1 tumors were significantly smaller 21 days following the initial injection ($P < 0.02$), at which point, they began to regrow. Interestingly, the more rapidly growing HEI193 tumors were more susceptible to the virus, and 38 days following the initial injection only scar tissue was found in the region of the tumor in 4/6 animals, whereas in the other two animals the tumor was still very small (one being $50\,mm^3$ and the other $90\,mm^3$ as measured with external calipers).

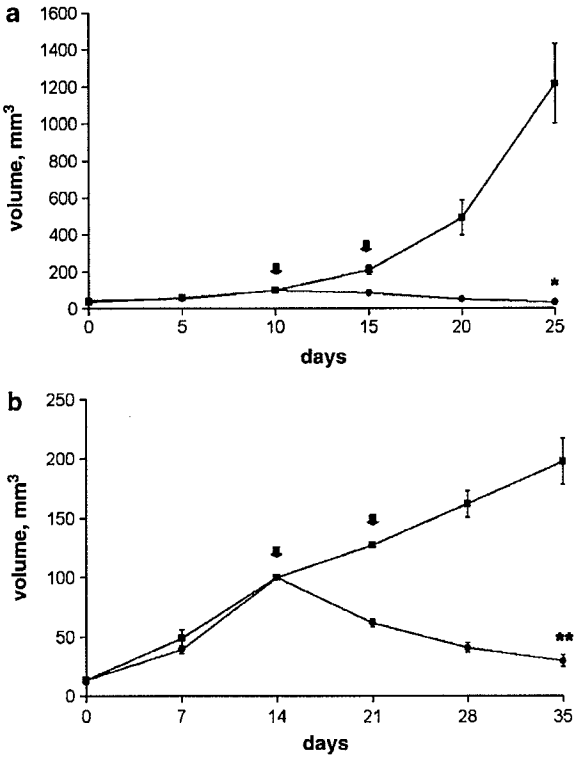

Figure 3 Growth of schwannoma tumors and response to G47Δ. HEI193 (**a**) and NF2S-1 (**b**) tumors were grown subcutaneously in nude mice ($n = 6$/group). When they reached a volume of $100\,mm^3$, they were injected directly with G47Δ virus (2×10^7 pfu; solid circles) or same volume of control vehicle (solid squares) two times as indicated by arrows. * indicates a significant difference between vehicle- and virus-injected tumors of $P < 0.01$ in (**a**) and ** of $P < 0.02$ in (**b**) at the final time point.

Discussion

Findings in this study

This represents the first successful attempt to generate schwannomas from cell lines in nude mice, with a critical factor being use of matrigel at the time of cell implantation. In addition to the immortalized human schwannoma line, HEI193 previously generated by Hung *et al.*,[20] we have now characterized an induced mouse schwannoma line NF2S-1, which is syngeneic with the Swiss strain background of the nude mice in which it arose. The human schwannoma line grew more rapidly, both in culture and as an implant, compared to the NF2S-1 line, and both were positive for S100, consistent with Schwann cell origin.[22] The growth rate of the mouse schwannoma line was in the range of that observed for spontaneous tumors in the NF2 transgenic model,[15] whereas the human schwannoma grew at a 10-fold faster rate. This

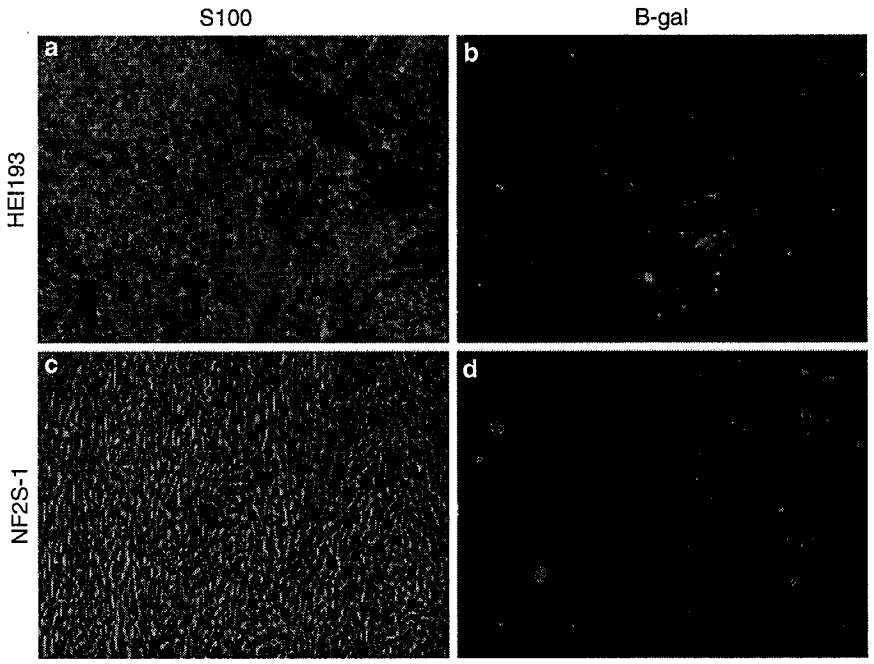

Figure 4 Immunostaining of virus-treated schwannoma tumors for S100 and lacZ. Subcutaneous tumors were removed 15 days (HEI193; **a** and **b**) or 21 days (NF2S-1; **c** and **d**) after the first injection of G47Δ. Cells in both types of tumors stained positively for S100 (**a** and **c**) and scattered cells within both tumors stained positively for *lacZ* (**b** and **d**). Magnification = × 10.

may reflect varying growth rate and range seen in NF2-associated human schwannomas, although such rapid growth is unusual in human schwannomas. As previously shown for spontaneous schwannomas in NF2 transgenic mice and human schwannoma tissue implants,[15] both schwannoma cell lines were highly infectable with the oncolytic virus G47Δ and both tumors regressed rapidly in response to direct injection of this vector. There was complete regression and no regrowth of most human HEI193 schwannomas after treatment, whereas the mouse NF2S-1 tumors resumed growth at the rate before treatment within 2 weeks after the last vector injection. This differential sensitivity of schwannomas to HSV therapy is consistent with: the higher burst size of G47Δ virus in HEI193 as compared to NF2S-1 cells; the faster growth rate of HEI193 tumors combined with the selectivity of G47Δ replication for rapidly dividing cells; and the increased susceptibility of human cells versus mouse cells to HSV-1 infection. These studies support the use of oncolytic G47Δ for treatment of rapidly growing schwannomas as a means of shrinking the tumor mass, while minimizing potential damage to associated nerves.

Advantages and disadvantages of current mouse models for schwannomas

A number of mouse models of schwannoma have been generated which have provided insights into the etiology and pathology of these tumors based on mutations in the tumor suppressor gene, *NF2*.[23,24] Giovannini *et al*.[10] generated transgenic mouse lines in which a dominant negative mutant form of merlin, which has been identified in some human NF2 patients, was expressed under the Schwann cell-specific P0 promoter. These transgenic mice developed Schwann cell-derived tumors between 9 and 20 months of age typically in the trigeminal ganglia, spinal ganglia and nerve endings in skeletal muscle. Based on MRI volumetric tracking of several tumors in these mice, the growth rate was estimated at about 0.5 mm^3/day.[15] In a second model, Giovannini *et al*.[12] characterized a conditional knock-out model of NF2 (NF2floxed), which developed schwannomas, Schwann-cell hyperplasias, cataracts and osseous metaplasias when crossed with transgenic mice expressing Cre recombinase under the P0 promoter, as well as meningiomas after neonatal intraventricular injections of adenovirus encoding Cre recombinase.[13] Furthermore, introduction of hemizygosity for the tumor suppressor gene *p53* in the NF2floxed × P0-Cre mice resulted in rapid generation of malignant peripheral nerve sheath tumors by the time of death, within 2 months after birth.[14] Subcutaneous tumors have also been generated in nude mice by implantation of fragments of human schwannoma tissue (removed at surgery), albeit the success rate was only about 17% and tumors grew slowly (about 5 mm^3/day;[15]). The present study provides a new model of schwannomas, which are formed in nude mice by injection of schwannoma cell lines. It offers particular advantages for assessing therapeutic agents because the tumors are placed in a reproducible, accessible site, and growth/regression can be monitored over time by external caliber measurements. In addition, for the mouse schwannoma cell line studies can potentially be carried out in a syngeneic, immune competent host.

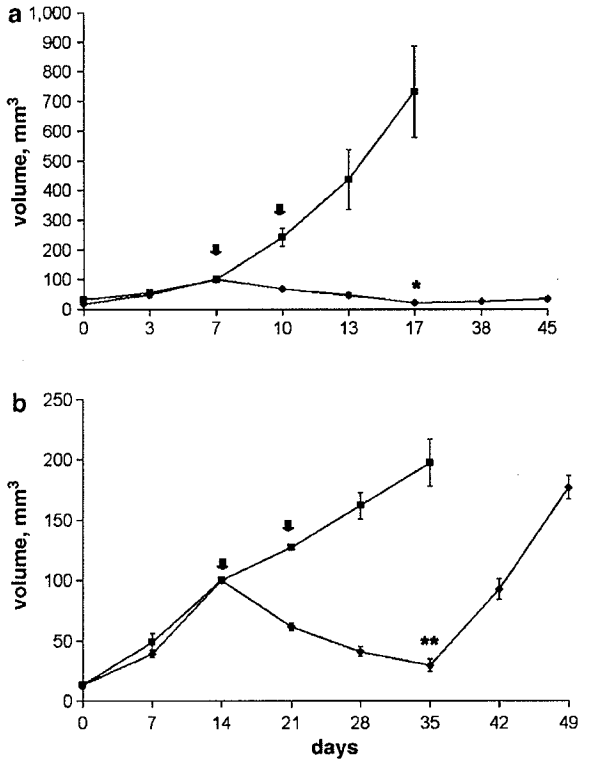

Figure 5 Extended evaluation of G47Δ-treated tumors. HEI193 (**a**) and NF2S-1 (**b**) tumors were established and injected with G47Δ as in Figure 3, the injections were carried out when the tumors were about 100 mm^3 in size at 7 and 10 days for HEI193 tumors and at 14 and 21 days for NF2S-1 tumors. In both cases, tumor size was reduced significantly, $P<0.002$ for HEI193 tumors on day 10 after initial treatment and $P<0.02$ for NF2S-1 tumors on day 21 after initial treatment.

Origin of mouse schwannoma

It is not clear how subcutaneous implantation of human schwannoma tissue from an NF2 patient resulted in generation of a schwannoma tumor from endogenous mouse Schwann cells. It is possible that growth factors or peripheral nerve sheath damage caused by implantation of the implant resulted in dedifferentiation and proliferation of Schwann cells at that site. These endogenous Schwann cells may then have undergone genetic changes that led to continuing proliferation. Their classification as schwannoma cells is based on positive staining for S100 (immunofluorescence) and GFAP (immunohistochemistry), as well as selective activation of the P0 promoter (in preparation).

Current treatment paradigms and risk/benefit of oncolytic HSV

The current treatment paradigm for human schwannomas is either surgical removal or radiosurgery, and because these tumors are benign, even reduction in tumor mass can be beneficial as there is no risk of metastasis. However, in some cases, especially cranial nerve schwannomas, resection may not be possible or may cause unacceptable damage. Therefore, oncolytic HSV vectors provide a new therapeutic option. These oncolytic vectors typically lack genes for ribonucleotide reductase and gamma 34.5 which allow them to propagate selectively in dividing cells, thereby killing them and generating more virus on site, with low toxicity to central or peripheral nerves.[18,25,26] The G207 HSV vector of this configuration has been tested in phase 1 clinical trials for brain tumors and found to have no serious adverse side effects.[27] The G47Δ vector used in the present study has the same backbone as G207, but is additionally mutated for ICP47, which blocks antigen presentation in infected cells,[28] resulting in increased antiviral and antitumor T-cell activity and enhanced propagation and therapeutic efficacy in a number of tumor cell types as compared to G207.[19,29] Given the high infectivity of schwannomas by HSV vectors and the efficiency of oncolytic HSV in reducing tumor mass, these vectors offer a promising alternative or combinatorial therapy in treating schwannoma tumors in humans.

Acknowledgements

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References

1 Short MP, Richardson EPJ, Haines JL, Kwiatkowski DJ. Clinical, neuropathological and genetic aspects of the tuberous sclerosis complex. *Brain Pathol* 1995; **5**: 173–179.
2 Baser ME, Evans DG, Gutmann DH. Neurofibromatosis 2. *Curr Opin Neurol* 2003; **16**: 27–33.
3 McClatchey AI, Giovannini M. Membrane organization and tumorigenesis—the NF2 tumor suppressor, Merlin. *Genes Dev* 2005; **19**: 2265–2277.
4 Jessen KR, Mirsky R. The origin and development of glial cells in peripheral nerves. *Nat Rev Neurosci* 2005; **6**: 671–682.
5 MacCollin M, Chiocca EA, Evans DG, Friedman JM, Horvitz R, Jaramillo D *et al.* Diagnostic criteria for schwannomatosis. *Neurology* 2005; **64**: 1838–1845.
6 Wilkes D, McDermitt DA, Basson CT. Clinical phenotypes and molecular genetic mechanisms of Carney complex. *Lancet Oncol* 2005; **6**: 501–508.
7 Trofatter JA, MacCollin MM, Rutter JL, Murrell JR, Duyao MP, Parry DM *et al.* A novel moesin-, ezrin-, radixin-like gene is a candidate for the neurofibromatosis 2 tumor suppressor. *Cell* 1993; **72**: 791–800.
8 Rouleau GA, Merel P, Lutchman M, Sanson M, Zucman J, Marineau C *et al.* Alteration in a new gene encoding a putative membrane-organizing protein causes neuro-fibromatosis type 2. *Nature* 1993; **63**: 515–521.
9 Martuza RL, Eldridge R. Neurofibromatosis 2 (bilateral acoustic neurofibromatosis. *N Engl J Med* 1988; **318**: 684–688.

10 Giovannini M, Robanus-Maandag E, Niwa-Kawakita M, van der Valk M, Woodruff JM, Goutebroze L *et al.* Schwann cell hyperplasia and tumors in transgenic mice expressing a naturally occurring mutant NF2 protein. *Genes Dev* 1999; **13**: 978–986.
11 Messerli SM, Tang Y, Giovannini M, Bronson R, Weissleder R, Breakefield XO. Detection of spontaneous schwannomas by MRI in a transgenic murine model of neurofibromatosis type 2. *Neoplasia* 2002; **4**: 501–509.
12 Giovannini M, Robanus-Maandag E, van der Valk M, Niwa-Kawakita M, Abramowski V, Goutebroze L *et al.* Conditional biallelic Nf2 mutation in the mouse promotes manifestations of human neurofibromatosis type 2. *Genes Dev* 2000; **14**: 1617–1630.
13 Kalamarides M, Niwa-Kawakita M, Leblois H, Abramowski V, Perricaudet M, Janin A *et al.* Nf2 gene inactivation in arachnoidal cells is rate-limiting for meningioma development in the mouse. *Genes Dev* 2002; **16**: 1060–1065.
14 Robanus-Maandag E, Giovannini M, van der Valk M, Niwa-Kawakita M, Abramowski V, Antonescu C *et al.* Synergy of Nf2 and p53 mutations in development of malignant tumours of neural crest origin. *Oncogene* 2004; **23**: 6541–6547.
15 Messerli SM, Prabhakar S, Tang Y, Mahmood U, Weissleder R, Bronson R *et al.* Treatment of schwannomas with an oncolytic recombinant herpes simplex virus in murine models of neurofibromatosis type 2. *Hum Gene Ther* 2006; **17**: 20–30.
16 Aghi M, Chiocca EA. Genetically engineered herpes simplex viral vectors in the treatment of brain tumors: a review. *Cancer Invest* 2003; **21**: 278–292.
17 Markert JM. Biologic warfare for a good cause: HSV-1 antitumor therapy. *Clin Neurosci* 2004; **51**: 73–80.
18 Mineta T, Rabkin SD, Yazaki T, Hunter WD, Martuza RL. Attenuated multi-mutated herpes simplex virus-1 for the treatment of malignant gliomas. *Nat Med* 1995; **1**: 938–943.
19 Todo T, Martuza RL, Rabkin SD, Johnson PA. Oncolytic herpes simplex virus vector with enhanced MHC class I presentation and tumor cell killing. *Proc Natl Acad Sci USA* 2001; **98**: 6396–6401.
20 Hung G, Li X, Faudoa R, Xeu Z, Kluwe L, Rhim JS *et al.* Establishment and characterization of a schwannoma cell line from a patient with neurofibromatosis 2. *Int J Oncol* 2002; **20**: 475–482.
21 Galve-Roperh I, Sanchez C, Cortes ML, del Pulgar TG, Izquierdo M, Guzman M. Anti-tumoral action of cannabinoids: involvement of sustained ceramide accumulation and extracellular signal-regulated kinase activation. *Nat Med* 2000; **6**: 313–319.
22 Kleihues P, Cavenee WK. *Pathology and Genetics of Tumours of the Nervous System.* IARC Press: Lyon, 2000.
23 Gutmann DH, Giovannini M. Mouse models of neurofibromatosis 1 and 2. *Neoplasia* 2002; **4**: 279–290.
24 McClatchey AI, Cichowski K. Mouse models of neurofibromatosis. *Biochem Biophys Acta* 2001; **1471**: M73–M80.
25 Mashour GA, Moulding HD, Chahlavi A, Khan GA, Rabkin SD, Martuza RL *et al.* Therapeutic efficacy of G207 in a novel peripheral nerve sheath tumor model. *Exp Neurol* 2001; **169**: 64–71.
26 Chiocca EA. Oncolytic viruses. *Nat Rev Cancer* 2002; **2**: 938–950.
27 Markert JM, Medlock MD, Rabkin SD, Gillespie GY, Todo T, Hunter WD *et al.* Conditionally replicating herpes simplex virus mutant, G207 for the treatment of malignant glioma: results of a phase I trial. *Gene Ther* 2000; **7**: 867–874.
28 Hill A, Jugovic P, York I, Russ G, Bennink J, Yewdell J *et al.* Herpes simplex virus turns off the TAP to evade host immunity. *Nature* 1995; **375**: 411–415.
29 Liu R, Varghese S, Rabkin SD. Oncolytic herpes simplex virus vector therapy of breast cancer in C3(1)/SV40 T-antigen transgenic mice. *Cancer Res* 2005; **65**: 1532–1540.